Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 212–216, January 1989
Genetics

Rapid typing of tandemly repeated hypervariable loci by the polymerase chain reaction: Application to the apolipoprotein B 3′ hypervariable region

(variable number of tandem repeats/polymorphism)

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Communicated by James V. Neel, September 29, 1988

ABSTRACT **The 3′ flanking region of the apolipoprotein B (apoB) gene contains a hypervariable region consisting of a variable number of tandemly repeated short A+T-rich DNA sequences (VNTRs). We present a general method that utilizes the polymerase chain reaction to rapidly and accurately type this and other VNTR loci. We use tailored oligonucleotides and thermostable *Taq* polymerase to amplify the targeted region. The amplification products are directly visualized after agarose gel electrophoresis. Twelve alleles were readily identified in a sample of 125 unrelated individuals. The alleles differ with respect to the length of the amplified gene region. This genetic variability is inherited in an autosomal codominant manner. DNA sequence data indicate that individual alleles differ in the number of repeat units and the sensitivity of the technique is such that alleles differing in length by only 32 base pairs are readily distinguishable. A system of nomenclature based on the number of repeat units is suggested; an allele containing 37 repeat units is designated 3′β37, one containing 35 units is 3′β35, and so on. The frequency distribution of the 12 apoB VNTR alleles is bimodal with peaks at 37 and 47 repeat units and a nadir near 43 repeat units. We estimate that the 3′ apoB VNTR locus has a heterozygosity index of 0.75 and a polymorphic information content of 0.73. It is a highly informative marker for genetic linkage studies on chromosome 2 and clinical and epidemiological studies involving the apoB gene. The high sensitivity and inexpensive nature of this technique make it superior to traditional Southern blot analysis for typing the 3′ apoB VNTR. The method described is also directly applicable for rapid typing of other VNTRs in the human genome.**

Genetic markers are important tools for the study of human disease. For coronary heart disease, the leading cause of morbidity and mortality in the United States (1), markers have been used in case control studies (2), in association studies using a sample of unrelated individuals (3, 4), and to examine the inheritance of traits in a sample of families (5). The genetic markers traditionally considered are contained at random loci and in genes thought to be involved in lipid metabolism and the disease process. They include serological types, protein isoforms, and restriction-site polymorphisms in specific DNA sequences. The utility of these and other genetic markers is determined in large part by the extent of allelic variability that exists at a particular locus.

Genetic polymorphisms resulting from a variable number of tandemly repeated short DNA sequences (VNTRs) have been described (6, 7). One VNTR is located ≈0.5 kilobases (kb) on the 3′ side of the last amino acid codon in the apolipoprotein B (apoB) gene (8, 9). Clinical and epidemiological studies have established a positive relationship of coronary heart disease and low density lipoprotein cholesterol with apoB levels (10). ApoB-100 is the major apolipoprotein in low density lipoprotein and is the principal ligand for the low density lipoprotein receptor. ApoB-100 cDNA and genomic sequences have been cloned and sequenced (11–16). ApoB-100 was found to be one of the largest monomeric proteins known containing 4536 amino acid residues. The apoB gene spans 42 kb and maps to the short arm of chromosome 2 (17). Two forms of the apoB protein, apoB-48 and apoB-100, are coded for by a single apoB gene. The molecular basis for the difference between these gene products has been described (18, 19).

Allelic variation at the apoB 3′ hypervariable region is detectable by Southern blot analysis using *Msp* I, *Eco*RV, *Hin*dIII, and other restriction enzymes and the 3′ end of the apoB cDNA as a hybridization probe. Studies using Southern blot analysis indicate that this region is complex and 5 alleles have been proposed (9, 20, 21). However, the unequivocal assignment of specific alleles to individual DNA bands is difficult because of minor differences in the size of the relatively large restriction fragments. In this communication we have analyzed this region of the apoB gene by the polymerase chain reaction (PCR) technique (22). This method allows for rapid and accurate typing of the VNTR locus at the 3′ end of the apoB gene. Our results indicate that this region of the apoB gene is one of the most polymorphic loci studied to date with a minimum of 12 alleles observed in a sample of 125 unrelated individuals. These alleles differ with respect to the number of short repeat units that are easily missed by traditional Southern blot methods. This locus contains a very large amount of allelic variation making it a highly informative marker for genetic linkage studies on chromosome 2 and for studies of cardiovascular disease risk factors. The method we describe is also applicable for typing allelic variation at other VNTR loci.

MATERIALS AND METHODS

Genomic DNA was purified from isolated buffy-coat cells by phenol extraction on an automated nucleic acid extractor (Applied Biosystems, Foster City, CA). The oligonucleotide primers were 20 nucleotides long and were synthesized on an Applied Biosystems model 380A DNA synthesizer. The sequences of the primers were chosen such that they flank the targeted region of the genome on the 3′ side of the apoB gene. Unique DNA sequences remained within the targeted region

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Abbreviations: VNTR, variable number of tandemly repeated short DNA sequence; apoB, apolipoprotein B; PCR, polymerase chain reaction.

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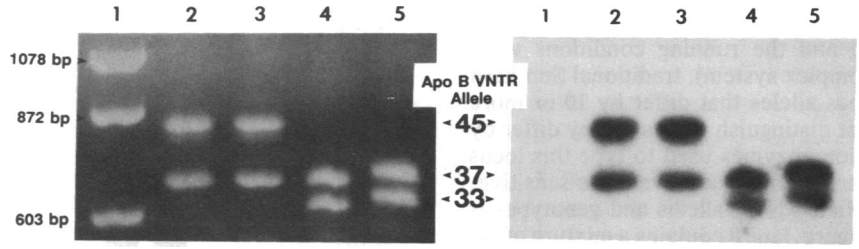

FIG. 1. Products of two independent PCRs for two individuals. (*Left*) PCR products analyzed on an ethidium bromide-stained agarose gel. (*Right*) An autoradiogram of the corresponding DNA blot hybridized to an end-labeled 20-base oligonucleotide within the targeted region on the 3′ side of the apoB gene. Lane 1 contains DNA size standards from ϕX174 digested with *Hae* III. The probe does not hybridize with sequences from the size standards. Lanes 2 and 3 are the PCR products from one individual, and lanes 4 and 5 are products from another. There is complete concordance between duplicate amplification products from the same individual.

allowing us to detect the amplified apoB VNTR by hybridization with complementary oligonucleotide probes. The sequence of the 5′ oligonucleotide used to prime the PCR was 5′-ATGGAAACGGAGAAATTATG-3′. The sequence of the 3′ PCR primer was 5′-CCTTCTCACTTGGCAAATAC-3′. The PCR was carried out in a final volume of 100 μl. One microgram of genomic DNA and 1 μg each of the oligonucleotides were used for each reaction. The four dNTPs were present each at a final concentration of 200 μM. The reaction buffer used was that recommended by the manufacturer. Prior to addition of the *Taq* polymerase, the reaction mixture was boiled for 7 min then allowed to cool briefly. Three units of thermostable *Taq* polymerase (Perkin–Elmer–Cetus, Norwalk, CT) was used to amplify the targeted sequence. Annealing and extension was carried out for 6 min at 58°C and denaturing was carried out for 1 min at 94°C. Twenty-six extension–denaturation cycles were carried out for each PCR.

Electrophoresis of the amplified DNA was carried out on 2% agarose gels at 70 V for 4–5 hr. The amplification products were visualized directly with ethidium bromide. To verify that the products were from the targeted sequence, the electrophoresed DNA was transferred to a charged nylon membrane (GeneScreen*Plus*, Dupont) according to the manufacturer's directions. A 20-base oligonucleotide (5′-TATGGAGGGAAATATTTTGC-3′), which corresponds to a unique sequence within the target region, was used as a hybridization probe. End-labeling was carried out with T4 polynucleotide kinase and [γ-^{32}P]ATP. Results obtained from the PCR technique were compared to those obtained from traditional Southern blot analysis. Seven micrograms of genomic DNA were digested with *Msp* I, electrophoresed on 0.8% agarose gels, and transferred to a charged nylon membrane (GeneScreen*Plus*, Dupont). The filters were hybridized to a 2.8-kb apoB cDNA probe corresponding to the 3′ end of the cloned apoB cDNA (23). The probe was labeled with ^{32}P by the random-priming method of Feinberg and Vogelstein (24).

The PCR products from two individuals were purified from agarose gels and subcloned into pGEM-4 by synthetic linkers. The PCR products were then sequenced in the double-stranded form by the dideoxynucleotide chain-termination technique of Sanger *et al.* (25). Both strands were sequenced in their entirety.

RESULTS

The products of four amplification reactions from two individuals are shown in Fig. 1 *Left*. Two bands were observed for amplification products from each individual and the banding patterns are concordant between two independent amplification reactions on DNA from the same individual. We have carried out numerous pairs of confirmatory PCRs and obtained complete concordance between the typing of all pairs. The products from one individual shown in lanes 2 and 3 has two bands ≈120 base pairs (bp) apart. The smaller fragment in lanes 2 and 3 is the same size as the larger fragment from amplification products of another individual shown in lanes 4 and 5. The amplified fragments are in the expected size range based on the position of the synthetic oligonucleotide primers and on the published sequence data (8, 9). Fig. 1 *Right* shows a Southern blot of the agarose gel in Fig. 1 *Left* hybridized with an internal synthetic oligonucleotide probe. The pattern of the amplified DNA fragments observed on the ethidium bromide-stained agarose gel is reproduced on the autoradiogram, confirming that the bands observed on the agarose gel are in fact from the targeted VNTR on the 3′ side of the apoB gene.

Typing the apoB 3′ VNTR with the PCR is much more sensitive than traditional Southern blot analysis. Fig. 2 shows the amplification products of five individuals whose alleles differ in length by 0, 2, 4, 12, or 10 repeat units. Also shown are the results of Southern blot analysis of genomic DNA from these same individuals digested with *Msp* I and probed with a labeled DNA fragment in the 3′ end of the apoB gene. The bottom 2.3- to 2.6-kb bands are polymorphic and

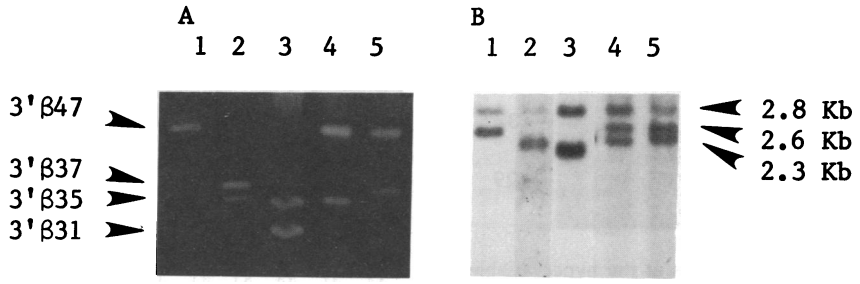

FIG. 2. The electrophoresed and stained apoB VNTR amplification products of five individuals (*A*) are shown next to the results from traditional Southern blot analysis of the apoB VNTR on the same individuals (*B*). For the Southern blot analysis genomic DNA was digested with *Msp* I and probed with a fragment in the 3′ end of the apoB structural gene (23). The individual in lane 1 is homozygous for allele 3′β37. Lanes 2, 3, 4, and 5 contain amplification products of heterozygous individuals with genotypes 37/35, 35/31, 47/35, and 47/37, respectively. The 2.8-kb band is from a monomorphic fragment located 5′ of the VNTR in the apoB gene.

encompass the 3′ apoB VNTR. Even under optimal conditions (i.e., the enzyme and the running conditions were chosen for typing this complex system), traditional Southern blot analysis distinguishes alleles that differ by 10 or more repeat units and does not distinguish alleles if they differ by 4 units or less. Restriction enzymes used to type this locus that generate even larger fragments are even less sensitive.

A collection of amplified VNTR alleles and genotypes is shown in Fig. 3. In this figure, lane 8 contains a mixture of the amplification products encompassing 12 different-sized alleles detected in a sample of 125 unrelated individuals. Lanes 1–7 show the banding patterns of genotypes containing each of the alleles. The fragment sizes are clearly distinguishable from one another. No individual exhibits more than two major PCR products. Based on their relative migration with respect to DNA size standards, the observed fragments range in size from ≈570 to 900 bp. Therefore, by calculation, each allele differs in length from its neighboring allele by ≈30 bp or two 15-bp repeat units. The alleles are named according to the number of repeat units they are inferred to have. Among the 12 alleles scored in a sample of 125 unrelated individuals, the relative frequencies of these apoB 3′ VNTR alleles are shown in Fig. 4. The most frequent allele is the 691-bp allele that contains 37 repeat units with a frequency of 0.428. The next most frequent allele is the 659-bp allele that contains 35 repeat units with a relative frequency of 0.204. Not surprisingly then, the most common genotype observed in this sample (28 out of 125) is the heterozygote consisting of these two common alleles. Surprisingly, the third most common allele has 47 repeat units and a relative frequency of 0.100. The other 9 alleles are less frequent in the population with an estimated frequency of <0.1. Alleles with 43 and 45 repeat units show a clear nadir in the frequency distribution. The possible etiology of this bimodal distribution is discussed below.

Fig. 5 shows the inheritance of several banding patterns in three families. In each family the parents are heterozygous for the typed alleles. In two of the three families four different alleles are present in the two parents. This bodes well for the potential of this VNTR locus typed by the PCR for linkage studies on chromosome 2. The 3′ apoB VNTR types are inherited in a manner consistent with an autosomal codominant trait as expected for detectable DNA variation.

To determine the exact size of the amplified region and to determine the basis for the size differences between alleles, we sequenced the amplification product of three alleles, one from an individual homozygous for the 3′β37 allele (an allele with 37 repeat units, see below for a discussion on nomenclature), and two alleles from an individual heterozygous for the 3′β37 and 3′β35 alleles. The DNA sequences of the PCR products of these three alleles are given in Fig. 6. The two alleles labeled 3′β37 are the size of the most common allele observed in the sample of 125 unrelated individuals. From the

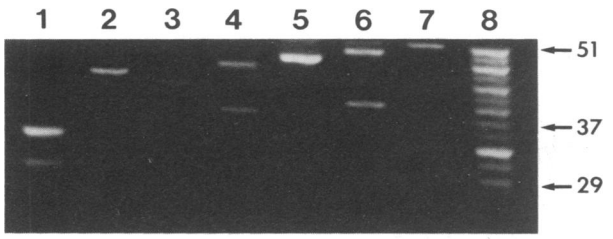

FIG. 3. Amplification products of alleles at the hypervariable region on the 3′ side of the apoB gene. Lane 8 contains a mixture of the 12 allele products identified in a sample of 125 unrelated individuals. The alleles are labeled 29–51 from smallest to largest. The allele numbers indicate the number of repeat units inferred for each allele. Lanes 1–7 show patterns of genotypes containing each of the 12 alleles.

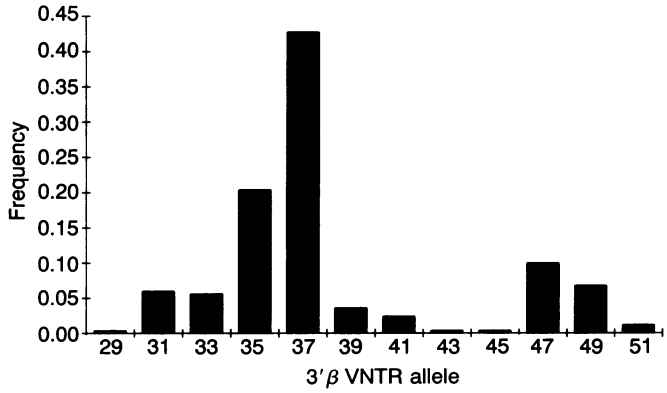

FIG. 4. Frequency distribution of the 12 3′ apoB VNTR alleles in a sample of 125 unrelated individuals. Individuals were associated without respect to their lipid profile and are all Caucasian adults of French ancestry.

DNA sequence, the amplification products of the 3′β37 alleles are 691 bp long. Each allele contains 37 repeat units that are mostly 14–16 bp long. Individual repeats begin with multiple thymines and are highly A+T-rich. Some repeats also contain a single cytidine or guanosine. The sequence listed first and in its entirety matches exactly the sequence of this region reported by Knott *et al.* (8). The second sequence listed in Fig. 6 is also designated 3′β37. This allele is the same length as the first allele although slightly different in sequence composition. The allele labeled 3′β35 and whose sequence is shown third in Fig. 6 contains two repeat units less than the other two sequenced alleles. The insertion/deletion of two repeat units is the cause of the differences in electrophoretic mobilities between 3′β35 and 3′β37 and between the other 10 3′ apoB VNTR alleles (Fig. 3). Each missing repeat unit is 16 bp long and the amplification product of the 3′β35 VNTR is 659 bp. Apart from the two missing repeat units, the sequence of the 3′β35 allele is otherwise identical to the first allele with 37 repeat units.

DISCUSSION

We have presented a method for rapidly and accurately typing a VNTR on the 3′ side of the human apoB gene. The method uses tailored oligonucleotides and thermostable *Taq* polymerase to amplify the targeted region. The amplification products are directly visualized by staining after agarose gel electrophoresis. This region is highly polymorphic; 12 alleles were observed in a sample of 125 unrelated individuals. The alleles differ with respect to the number of A+T-rich repeat units. The method described here enables one to distinguish alleles that are indistinguishable by traditional Southern blot analysis. The apoB 3′ VNTR is detectable by a number of

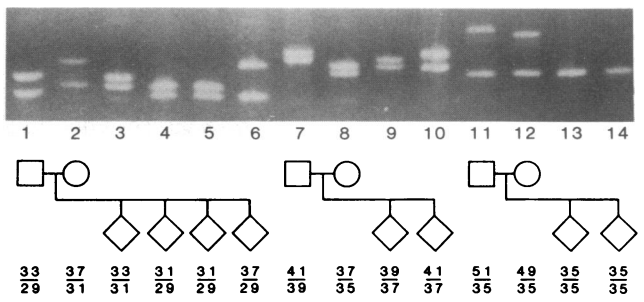

FIG. 5. Mendelian inheritance of 3′ apoB VNTR alleles in three families. The pedigrees for each family are drawn below the lanes of the ethidium bromide-stained agarose gel. The allele designations correspond to the number of repeat units inferred for each allele.

FIG. 6. DNA sequence of three 3′ apoB VNTR alleles. Two of the alleles (3′β37) have the same electrophoretic mobility on an agarose gel. One allele (3′β35) migrates slightly faster than the other two. The sequence of one of the 3′β37 alleles is presented in its entirety. The sequence of the other 3′β37 allele is represented by dots where the bases are identical and by the alternative base where they are different. Differences have been confirmed by sequence analysis of multiple cloned amplification products. The PCR products of the 3′β37 alleles are 691-bp long. The majority of the A+T-rich repeat units are 14–16 bp long and are separated by blank spaces. Two of the repeat units are only 11 and 12 bp long. The 3′ apoB VNTR allele 3′β35 has two 16-bp repeat units missing from its sequence relative to the 3′β37 alleles. The missing sequences are highlighted by brackets in the figure. Beside the two missing repeat units, the sequence of 3′β35 is concordant with the sequence of the 3′β37 allele whose sequence is given first and in its entirety.

enzymes, including *Msp* I, *Hin*dIII, and *Eco*RV, and a probe from the 3′ end of the apoB gene. Depending on the enzyme used and the quality of the blot, the alleles must differ by multiple repeat units before they are distinguishable by traditional methods (Fig. 2). Utilizing the PCR with appropriate priming oligonucleotides allows one to separate alleles that differ by only 32 bp. Another important feature of the method is the ability to set up and complete the amplification reaction and score the 3′ apoB VNTR on the same day. In addition, each PCR requires only 1 μg of genomic DNA, which is an order of magnitude less than typing by Southern blot analysis. Further, we have successfully performed PCRs on partially degraded DNA and have accurately typed the apoB VNTR from their amplification products, which would have been impossible with Southern blot analysis. Finally, the method does not require a cloned apoB cDNA probe and radioactivity for typing. Any laboratory interested in this and similar polymorphic markers can perform these analyses without resorting to molecular cloning technology and equipment.

The alleles defined by this approach are readily discernible on an ethidium bromide-stained agarose gel. Although the DNA bands migrate very close to one another, Fig. 3 shows that the 12 alleles form a ladder with nonoverlapping rungs. Discriminating similar alleles between samples that are not run in close proximity to one another (e.g., on separate gels) can be difficult. When in doubt, we suggest rerunning samples in neighboring lanes on the same gel. To facilitate typing we can make available DNA of a known type for use as standard markers. Furthermore, one can take only 1% of the initial amplification product and reamplify by repeated PCRs producing essentially an unlimited supply of any well characterized or important sample. Faint bands occasionally appear in the amplification product. These bands are likely attributable to less specific priming of the synthetic oligonucleotides and have not caused difficulties typing the apoB VNTR region. If necessary, hybridization of bands to an internal end-labeled oligonucleotide will confirm the authenticity of the desired PCR product (Fig. 1).

We suggest a system of nomenclature for the 3′ apoB VNTR alleles that is based on the number of repeat units. An allele at this locus with 37 repeat units is labeled 3′β37 and so forth. Alleles corresponding to 3′β29 through 3′β51 are shown in Fig. 3. Using this system, alleles 1, 2, 3, and 4 of Huang and Breslow (9) correspond to 3′β35, 3′β35, 3′β37, and 3′β47, respectively. The allele sequenced by Knott *et al.* (8) corresponds to 3′β37. This nomenclature is independent of the particular method employed. It should promote some coherence in the field and will allow for direct comparisons of results between laboratories.

Based on the allele frequencies observed in this sample, we estimate that this genetic marker has an index of heterozygosity of 0.75 and a polymorphic information content of 0.73. It is one of the most polymorphic genetic loci in man. The 3′ apoB VNTR is an important marker for genetic linkage studies on chromosome 2 and for clinical studies utilizing markers at or near the apoB gene. In light of reports documenting the potential of the PCR to amplify both very small amounts of DNA and DNA isolated from diverse sources (26), the utility of PCR typing of the 3′ apoB and other VNTR loci takes on added importance.

The high degree of variability at this locus and other VNTR loci is presumably due to unequal recombination resulting from mismatching of the repeat units or from replication slippage. The frequency distribution of the number of repeat units in a population and the maintenance of this distribution are affected by the rate that new alleles are generated and the rate that random genetic drift and natural selection remove alleles from the population. A report on a select sample of VNTR loci typed by Southern blot analysis suggests that their rate of unequal recombination may reach as high as 5% per locus per generation (27). Typing carried out in families can be used to directly estimate the rate of unequal recombination at the 3′ apoB VNTR locus. Whatever their mutation rate, the bimodality of the frequency distribution of the apoB VNTR alleles was unexpected. However, examination of the frequency distributions of VNTR alleles reported by Jeffreys *et al.* (27) and for the insulin VNTR (28) indicates that they are also multimodal. Characteristics of the mispairing and unequal recombination of the VNTRs and the nonidentity of the individual repeat units are likely giving rise to this unusual frequency distribution.

It is evident from direct DNA sequence determination that the exact sequence of the repeat units among alleles of the same size (e.g., the two 3′β37 alleles in Fig. 6) is not necessarily identical. The arrangement of the repeat units may also be different and may affect the process of equal and unequal recombination. The shorter allele in Fig. 6, 3′β35, is missing two 16-bp repeat units relative to the 3′β37 alleles. Comparing the sequence of the first 3′β37 allele and the 3′β35

allele reveals that the missing repeat units in the latter are separated by a single repeat unit. It is tempting to speculate on the event(s) that gave rise to one allele from others. However, without knowledge about the exact alleles that gave rise to the 3′β35 or the 3′β37 alleles, any hypotheses on the underlying molecular events would be highly speculative. A detailed characterization of identified unequal recombinants and their parental alleles is necessary to shed light on the underlying mechanisms.

From case-control studies, DNA variability in the apoB 3′ VNTR detected by Southern blot analysis has been reported to be associated with myocardial infarction (2). It is evident from the results presented here that the sensitivity of traditional Southern blots does not allow a clear distinction between many of the VNTR alleles (Fig. 3). It is likely that associations of disease states with specific apoB alleles detected by the more sensitive PCR method will be stronger than the associations reported from studies using less sensitive methods (2, 20). Future case control and epidemiological studies on the association of apoB 3′ VNTR alleles with various normal and pathological traits should utilize the more accurate as well as sensitive PCR typing technique rather than traditional Southern blot analysis.

Other VNTR loci have been reported in the human genome. Some of these are near structural genes implicated in human disease, such as apoB (reported here), insulin (28), α-globins (29), and the *Hras* oncogene (30). The method described in this study utilizing the PCR to type hypervariable loci is directly applicable to these other loci. As for apoB, additional allelic variability will likely be uncovered at these regions because of the sensitivity of this technology over traditional Southern blot analysis. Application of the rapid typing method will enhance the value of these genetic loci allowing for more efficient determination of genetic linkage and more sensitive clinical and epidemiological studies of diabetes, hemoglobinopathies, and cancer.

E.B. thanks his colleagues at the Center for Preventive Medicine in Nancy, France, for their continued involvement and support of this research. This work is supported by grants from the National Institutes of Health [RR-07148 and HL-40613 (to E.B.) and HL-27341 (to L.C.)] and a grant from the March of Dimes Birth Defects Foundation (to L.C.).

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